IMMOBILITY OF THE CHROMOPHORE IN BACTERIORHODOPSIN

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1. Introduction

The purple membrane of *Halobacterium halobium* contains a single protein, bacteriorhodopsin, which has one molecule of retinal covalently bound to a lysine residue. Light absorption by the chromophore initiates a photochemical cycle which is accompanied by proton translocation from the cytoplasm to the medium and thereby produces an electrochemical gradient across the cell membrane [1]. The way in which the chromophore interacts with the protein is not yet understood well. In such a stage, it is of importance to answer the question whether the chromophore has any rotational freedom in the binding site.

Earlier studies of transient absorption dichroism of the purple membrane have shown that rotational motions of the protein in the membrane are absent in the time range from 10^{-4}–10^{3} s [2–4]. However, the reported values of absorption anisotropy are considerably lower than the theoretical maximum. This suggests a possibility that the chromophore and/or the protein has a freedom of rotation whose relaxation time is in the μs or shorter time region [5]. To clarify this point, we have carried out two independent experiments in which the rotational motions with 10^{-9}–10^{-2} s relaxation times can be monitored. One was the ns time-resolved fluorescence depolarization [6] and the other the transient absorption dichroism [7]. In the former experiment, we investigated $NaBH_4$-reduced purple membrane in which the chromophores were converted to fluorescent derivatives but the hexagonal crystalline structure was maintained [1]. The results show that the rotational motion of the chromophore as well as the protein in the purple membrane is completely absent in the time range examined.

2. Materials and methods

Purple membrane fragments were prepared from *Halobacterium halobium* R_1M_1 according to [8].

Purified purple membrane fragments were reduced with 1% $NaBH_4$ at 0°C under illumination with visible light (520–600 nm) from a projector [9]. The reduced product, bR^{red}, was washed and resuspended in 50 mM borate (pH 8.9). For the preparation of the UV-converted form of bR^{red}, the reduced purple membrane suspended in 10 mM phosphate (pH 7.0) was irradiated with UV-light under nitrogen until the characteristic absorption spectrum [9] fully developed.

Fluorescence decay was measured with a single-photoelectron counting apparatus [10]. The ns light pulses were provided by free-running discharge in H_2-gas (12 atm). The principal components of polarized fluorescence decay $I^{\parallel}(t)$ and $I^{\perp}(t)$ were simultaneously measured with two photomultiplier tubes (Hamamatsu R943-02). Fluorescence anisotropy decay was computed as follows:

$$r_F(t) = (I^{\parallel}(t) - I^{\perp}(t))/$$

$$(I^{\parallel}(t) + 2\,I^{\perp}(t)) \equiv D_F(t)/S_F(t)$$

Steady-excitation fluorometry was made with the same optical system, except that a Xenon lamp was used as an excitation source.

The principle of the transient absorption dichroism is described in [7]. After the sample was excited by a linearly polarized flash (duration 0.3 μs) from a dye laser, dichroism of transient absorption change was

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probed with a measuring light travelling perpendicularly with respect to the excitation beam. The absorption anisotropy was calculated from the principal components of absorption change $A^{\parallel}(t)$ and $A^{\perp}(t)$:

$$r_A(t) = (A^{\parallel}(t) - A^{\perp}(t))/$$

$$(A^{\parallel}(t) + 2A^{\perp}(t)) \equiv D_A(t)/S_A(t)$$

Here, the light intensity of the exciting flash was attenuated sufficiently, so that the fraction of the excited chromophores was <1%. Under this condition, distortion of the absorption anisotropy due to the excitation saturation [11] could be minimized.

3. Results and discussion

3.1. *Immobility of the chromophore in the binding site*

Fig.1 shows typical fluorescence kinetics of the

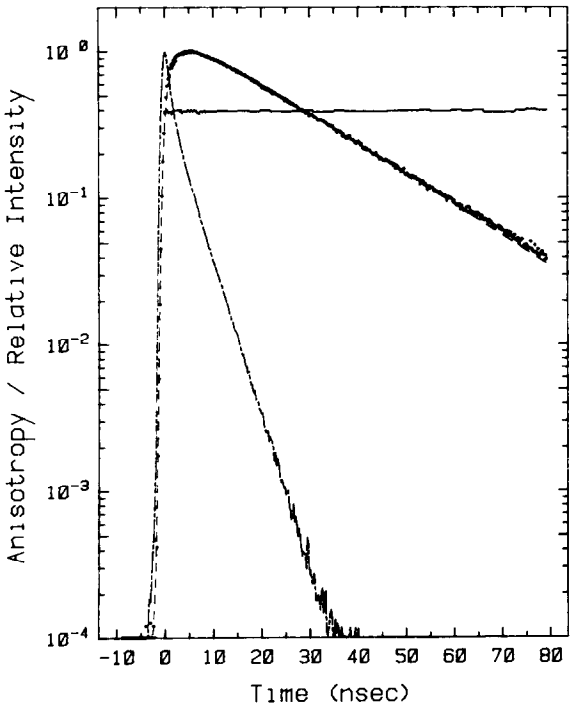

Fig.1. Decay curves of the total fluorescence intensity $S_F(t)$ (dots) and the fluorescence anisotropy $r_F(t)$ (solid line) of the UV-converted form of bR^{red} in 10 mM phosphate buffer (pH 7.0) at 10°C. The chain line represents the response function $g(t)$ of the apparatus. The dashed line is the convolution product of $g(t)$ and a single exponential intensity decay with a decay constant of 20.9 ns. Excitation was at 381 nm and emission above 460 nm was collected.

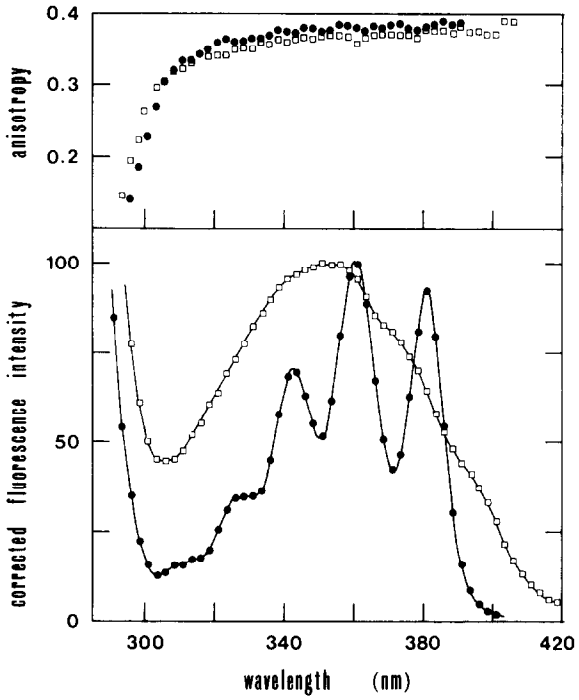

Fig.2. Excitation spectra of the total fluorescence intensity (bottom) and of the time-average fluorescence anisotropy (top) of bR^{red} in 50 mM borate at pH 8.9 (□) and of the UV-converted form in 10 mM phosphate at pH 7.0 (●) at 10°C. Emission above 460 nm was collected.

UV-converted form of bR^{red} at 10°C. The predominant feature in the figure is the high fluorescence anisotropy which remains constant over the 80 ns time range examined. The anisotropy value of 0.385 ± 0.005 (SD for 8 samples) is close to the theoretical maximum of 0.4, and by far the highest among many systems investigated so far in this laboratory at room temperature. The steady-state excitation anisotropy spectrum of the UV-converted form of bR^{red} shows that the remarkably high anisotropy value extends over the entire near-UV absorption band (●) as seen in fig.2. Moreover, the high anisotropy value was observed above the physiological temperature; the time-average anisotropy did not change detectably (<0.005) when the temperature of the sample was varied from 4–50°C.

The high anisotropy value indicates that the transition moment for the near-UV absorption band and the moment for the fluorescence emission are almost parallel with each other. In [9,12], the chromophore in the UV-converted form was all-*trans* retroretinyl attached to the protein, presumably via the same

lysine residue that forms the retinylidene linkage in the native bacteriorhodopsin. Thus the transition moments above are expected to lie along the polyene chain of the chromophore. The fact that the anisotropy remained constant after the pulsed excitation suggests that rotational motion of the chromophore around an axis perpendicular to the polyene chain is completely absent at least in the ns time range. The slight difference between the observed anisotropy of 0.385 and the theoretical maximum of 0.4 could be ascribed to a small deviation of the direction of the emission transition moment from that of the absorption transition moment. In this case, the angle between the two moments would be ~9°. Another probable explanation, however, is that the difference resulted from the scattering of the excitation and fluorescence lights in the sample.

The complete immobilization of the chromophore (around an axis perpendicular to the polyene chain) in the native purple membrane was confirmed by transient absorption dichroism measurements. Fig.3 shows the kinetics of absorption changes upon flash irradiation of an aqueous suspension of purple membrane fragments. The absorption anisotropy exhibited a high value immediately after the irradiation and decayed with a relaxation time of 17 ms (at 25°C). When the experimental curve was extrapolated to zero time, the limiting anisotropy of 0.395 ± 0.005 was obtained. This value is also very close to the theoretical maximum (0.4), and much higher than the value of 0.2 in [13] and those in [2,3]. One of the reasons why they observed the low values was that, in their flash photolysis experiments, the intensity of flash light was so strong that a saturation of the chromophore excitation occured [11]. In [17] a high limiting anisotropy of 0.37 ± 0.02 was also obtained by reducing the flash intensity. This result clearly shows that the rotational motion of the chromophore is completely absent in the ms or shorter time range. In addition, the decay of absorption anisotropy seen in fig.3 exactly corresponded to the rotation of whole purple membrane fragments, as judged from the result of the electric dichroism experiments [14]. Thus we conclude that the chromophore is completely immobilized within the purple membrane in the time range $<10^{-2}$ s.

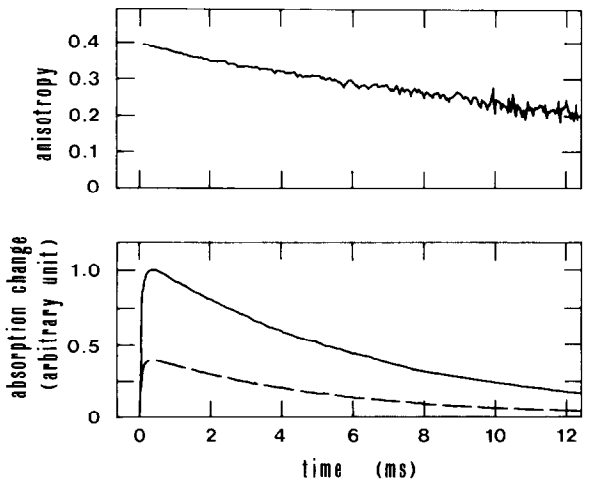

Fig.3. Decay curves of the total (solid line) and difference (dashed line) absorption change at 570 nm obtained upon flash irradiation at 488 nm of an aqueous suspension of purple membrane fragments at 25°C (lower panel). The anisotropy decay curve is presented in the upper panel.

3.2. *The chromophore in the excited state*

Addition of KI up to 0.1 M did not induce any change in the fluorescence lifetime of the UV-converted form of bR^{red}. The absence of dynamic quenching by KI indicates that the chromophore is completely isolated from the solvent. On the other hand, the fine structure in the excitation spectrum of the UV-converted form disappeared in its emission spectrum (fig.2,4), suggesting that the 'configuration' of the chromophore (and amino acid residues of the protein) in the excited state differs considerably from that in the ground state. This is also suggested by a dramatic discrepancy between the intrinsic fluorescence lifetimes calculated by the following two methods [15]. On the basis of the integrated strength of the near-UV absorption band, we expect that the radiative lifetime would be ~4 ns. Whereas, from the observed fluorescence lifetime (20.9 ns) and quantum yield (0.24), the intrinsic lifetime is expected to be ~87 ns. Since the direction of the transition moment for emission is almost parallel to that of the moment for absorption, a possible change of the configuration of the chromophore would be a kind of twist along the polyene chain.

The extent of the coplanarity between the ionone ring and the polyene chain is a factor determining the shape of the absorption spectrum of the retinal chromophore [8]. A conformational change similar to the twist may occur during the photochemical cycle of the native chromophore, since the intermediate M_{412} is known to show a fine structure in the absorption spectrum [16].

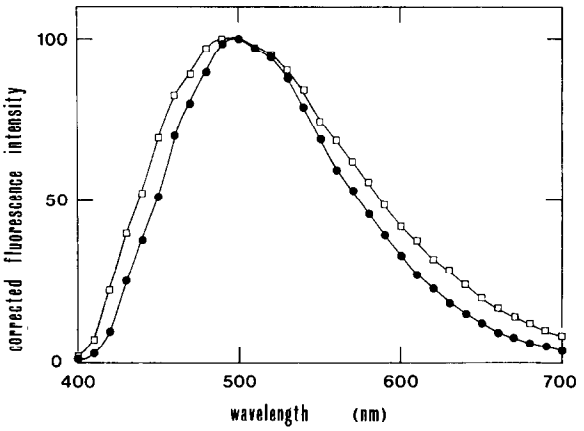

Fig.4. Emission spectra of bR^{red} in 50 mM borate at pH 8.9 (□) and of the UV-converted form in 10 mM phosphate at pH 7.0 (●). Excitation was at 360 nm for bR^{red} and at 381 nm for the UV-converted form.

3.3. *Non-UV-converted form of bR^{red}*

In contrast to the UV-converted form, the reduced (non-UV-converted) purple membrane, bR^{red}, gave a slowly decaying component with a very small amplitude (<0.03) in the 80 ns time range of the time-resolved fluorescence anisotropy (not shown). Over the near-UV band except for the longer-wavelength edge, the time-average anisotropy of bR^{red} was slightly smaller (~0.01) than that of the UV-converted form (fig.2). This may be due to excitation energy transfer among neighboring chromophores, since the bR^{red}, under the solvent conditions so far investigated, exhibited the excitation and emission spectra which overlapped with each other significantly (fig.2,4).

Rapid energy transfer among neighboring chromophores would lead to almost complete depolarization of fluorescence, as they are located around an axis of 3-fold rotational symmetry and tilted ~70° from the axis [1]. Thus the rather high anisotropy (0.370 ± 0.01 at 360 nm excitation) of bR^{red} indicates that the energy transfer is very slow as compared to the fluorescence lifetime (5–15 ns). The distance at which the efficiency of the energy transfer would be 50% at random orientation [18] was estimated to be 15–20 Å from the spectroscopic data. The neighboring chromophores are sufficiently separated from each other and oriented in such a manner that the chromophore-chromophore interaction may be thus close to the minimum. This feature for the distribution of the chromophore in the purple membrane is not compatible with that proposed in [19]. Details will be published elsewhere.

Conclusion

It was shown, from two independent experiments, that the rotational motion of the chromophore (around an axis perpendicular to the polyene chain) in the purple membrane was completely absent in the time range $<10^{-2}$s. The rigid packing of the main body of the chromophore in a pocket of the protein conceivably ensures efficient light-energy transduction, by imposing tight coupling between the photochemical events in the chromophore and probable conformational changes in the protein.

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